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An indirect competitive enzyme-linked immunosorbent assay for determination of norfloxacin in waters using a specific polyclonal antibody

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ABSTRACT

A specific polyclonal anti-norfloxacin antibody was obtained, and a sensitive indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed for determining trace amounts of norfloxacin in various waters. Good linearity was achieved in the range from 0.1 to 10 $\mu g L^{-1}$. The average IC_{50} value was determined to be 2.2 $\mu g L^{-1}$ and the limit of detection was 0.016 $\mu g L^{-1}$ at a signal-to-noise ratio of 3 in phosphate-buffered saline buffer. Recoveries of norfloxacin at various spiking levels ranged from 74 to 105% in groundwater, surface water, treated and untreated wastewater samples, with relative standard deviations of 3–5%. The assay was applied for determining norfloxacin in municipal wastewater, surface water, and groundwater collected in a metropolis of China. Raw wastewater samples were only submitted to filtration and pH adjustment while the other water samples were pre-concentrated by solid phase extraction prior to the icELISA assay. Good agreement of the results obtained by the icELISA and liquid chromatography tandem mass spectrometry further confirmed the reliability and accuracy of the icELISA for rapid detection of norfloxacin in waters.

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1. Introduction

Norfloxacin is a fluoroquinolone antibacterial agent that is extensively used in both human and veterinary medicine. In recent years, norfloxacin ranked as the second most prescribed fluoroquinolone antibacterial just next to levofloxacin in China. Norfloxacin, ofloxacin and ciprofloxacin have been so far the most frequently detected fluoroquinolones in the environment as summarized by Kümmerer [1]. It is well known that antibacterials in the environment may promote the development of antibacterial resistance in organisms [2,3]. The non-target toxicity of antibacterials, including norfloxacin, has also been documented in literature [4,5]. Although acute toxicity of norfloxacin at the environmentally relevant levels has not been reported, environmental monitoring for low level of norfloxacin in the environment is indispensable considering the unknown potential ecological risks.

A number of works have been conducted to determine fluoroquinolones in various environmental matrices including water, mainly based on high performance liquid chromatography (LC) separation coupled to various detectors including mass spectrometry [6–8], tandem mass spectrometry (MS/MS) [9–13], fluorescence detection [14–17] and ultraviolet (UV) detection [18,19]. However, these technologies generally required complicated sample preparation and expensive equipment.

Enzyme-linked immunosorbent assay (ELISA) has been extensively used for screening of veterinary drug residues including fluoroquinolones in food and animal tissues (e.g., muscle, kidney, liver, serum, milk, and egg) due to its sensitivity, specificity, rapidity, and simplicity [20–22]. Nevertheless, using ELISA for determination of fluoroquinolones and other pharmaceuticals in water samples has still been rarely reported [23].

Norfloxacin has also been widely detected in wastewater and river water in China [24]. The objective of this study is to develop a rapid, reliable, and sensitive method for detecting norfloxacin in water. A specific polyclonal antibody against norfloxacin was achieved and a sensitive indirect competitive ELISA (icELISA) method was subsequently developed through optimizing concentration of coating antigen, dilution of antibody, incubation time, etc. The method was applied to determine norfloxacin in treated wastewater, municipal wastewater, urban river water, lake water, and groundwater sampled from Guangzhou, a metropolis in South China. The icELISA results were further compared with those by LC–MS/MS analysis.

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2. Experiment

2.1. Chemicals and materials

N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC. HCl), ovalbumin (OVA), bovine serum albumin (BSA), *N*,*N*-hydroxysuccinimide (NHS), norfloxacin (NFX), ofloxacin, ciprofloxacin, enrofloxacin, pefloxacin mesylate dihydrate, enoxacin, marbofloxacin, lomefloxacin hydrochloride as well as both Freund's complete adjuvant (cFA) and incomplete adjuvant (iFA) were purchased from Sigma–Aldrich Company (St. Louis, MO, USA). Goat anti-rabbit IgG-horseradish peroxidase conjugate (GaRIgG-POD) was purchased from Pierce (Rockford, IL, USA). *O*-Phenylenediamine (OPD) was purchased from Solarbio (Beijing, China). *N*,*N*-Dimethylformamide (DMF), Tween-20 and hydrogen peroxide (H_2O_2, 30%) were purchased from Donghong Chemical Company (Guangzhou, GD, China).

Blocking buffer was purchased from Kem-En-Tec Diagnostics Company (Taastrup, Denmark) and was used as accepted. Other buffers were prepared with ultra-pure water. The coating buffer was 0.05 mmol L^{-1} sodium carbonate buffer (pH 9.6). Phosphate-buffered saline (PBS, pH 7.4) consisted of 1.47 mmol L^{-1} KH_2PO_4, 8.48 mmol L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 2.70 mmol L^{-1} KCl and 137 mmol L^{-1} NaCl. The washing buffer (PBST) was a PBS buffer with 0.05% (v/v) Tween-20. The substrate buffer (pH 5.0) was 0.1 mol L^{-1} phosphate/citrate buffer. The halting solution was 2 mol L^{-1} H_2SO_4. The substrate solution was prepared by dissolving 10 mg of OPD in 25 mL of substrate buffer. Ten microliters of H_2O_2 was added into the substrate solution 3 min prior to use.

2.2. Instrumentation

icELISA was carried out in 96-well microtitre plates (Nunc, Roskilde, Denmark). Immunoassay absorbance was read by a multiscan MK3 microplate reader (Thermo Fisher Scientific, Vantaa, Finland). The microplate was washed using a Thermo Fisher Scientific 4MK2 wellwasher. A Helios Alpha spectrometer (Thermo Spectronic, Cambridge, England) was used to collect UV data.

2.3. Synthesis of immunogen and coating antigen

An *N*-hydroxysuccinimide active ester method [25] was used for the synthesis of immunogen NFX-BSA and coating antigen NFX-OVA. Briefly, for the preparation of NFX-BSA, NFX (9.6 mg), NHS (17.25 mg), and EDC HCl (57.5 mg) were sequentially added into 3 mL of DMF. The mixture solution was magnetically stirred (420 rpm) for 24 h at room temperature in darkness. The mixture was then added to BSA solution that was prepared by dissolving 750 mg of BSA in 8 mL of PBS buffer. The reaction mixture was stirred for another 3 h followed by dialysis (molecular weight cut-off 10,000 Da) against PBS buffer for 3 days. The PBS buffer was renewed 3 times per day to remove the uncoupled free hapten. The obtained NFX-BSA immunogen was freeze dried and stored at −20 °C. The coating antigen NFX-OVA was prepared in a similar manner. UV absorbance spectrum was used to confirm the conjugation.

2.4. Antiserum production

Two female New Zealand white rabbits were subcutaneously immunized with the immunogen NFX-BSA at multiple sites. The initial immunization was performed by injecting 1.5 mg of NFX-BSA dissolved in 0.5 mL of sterilized physiological saline solution (PSS) and emulsified with 0.5 mL of cFA. Five booster immunizations were conducted by injecting 0.75 mg of NFX-BSA in 0.5 mL of PSS and 0.5 mL of iFA at 17-day intervals. The sixth booster (0.75 mg of NFX-BSA in 1 mL of PSS) was done 10 days later. From the third booster onward, serum titers were determined by ELISA seven days after each immunization to monitor the quality of the antisera from the immunized rabbits. Antiserum was obtained by centrifuging the blood at 10,010 × *g* for 10 min following clotting for 1 h at 4 °C.

Seven days after the last booster, 50 mL of blood was collected from the jugular vein of each rabbit. The antiserum was isolated by centrifuging the blood and was further purified with an IgG purified kit (Millipore, Billerica, MA, USA). The obtained antiserum was freeze dried and stored at −20 °C. The serum from the rabbit that showed the highest titer and sensitivity was used in this work.

2.5. Indirect competitive ELISA

One hundred microliters of NFX-OVA solution (1.6 μg L^{-1} in coating buffer) was coated in each well of a 96-well microtitre plate. The plate was incubated for 100 min at 37 °C and was washed with 350 μL of PBST for four times using the wellwasher. Kem-En-Tec synthetic blocking buffer (250 μL) was then added into each well and the plate was incubated for 8 min at room temperature. After the plate was washed as described before, 50 μL of diluted antibody (1:128000 in PBS) and norfloxacin standard solutions or samples were subsequently added, leading to the final incubation volume of 100 μL per well. Following additional incubation for 1 h at 37 °C, the plate was washed again. GaRIgG-POD was then added (1:10000 in PBS; 100 μL per well) and the plate was incubated at 37 °C for 1 h. For color development, 100 μL of substrate solution was added into each well. Six minutes later, the reaction was stopped by adding 50 μL of halting solution (2 mol L^{-1} H_2SO_4) into each well. The absorbance was measured by the microplate reader at 492 nm. The results were presented as inhibition = B/B_0, where B and B_0 were absorbance of the wells with and without competitor, respectively. A calibration curve was constructed in the form of B/B_0 versus Log C at norfloxacin concentrations of 0.01, 0.1, 0.5, 1, 5, 10, and 100 μg L^{-1}. Concentrations of norfloxacin in samples assayed in the same plate could then be determined using the standard curve.

2.6. Sensitivity and specificity of the assay

IC_{50} value (inhibitor concentration at B/B_0 ratio of 0.5) and cross-reactivity (CR) were determined to evaluate the sensitivity and specificity of the assay. Competitive immunoassays were performed using seven other fluoroquinolone compounds including ciprofloxacin, enrofloxacin, enoxacin, lomefloxacin, marbofloxacin, ofloxacin and pefloxacin. These compounds were chosen basically because they are structurally related to norfloxacin (Table 1). In addition, some of them, such as ciprofloxacin and ofloxacin have also been widely detected in the environment as norfloxacin [1]. The tested fluoroquinolones (0.01–10,000 μg L^{-1}) were deployed to the icELISA procedure as described above for norfloxacin. The CR values were then calculated as CR (%) = ($IC_{50\text{-norfolxacin}}/IC_{50\text{-tested compound}}$) × 100.

2.7. Sample collection and preparation

Water samples were collected from Guangzhou, a metropolis in South China. Three raw wastewater samples and one treated wastewater sample were collected from a sewage treatment plant that serves a population of around 2.5 million. Surface water samples were collected from the Pearl River and an urban lake. A groundwater sample was collected from a well. All the samples were stored in amber glass bottles without headspace and placed on ice packs during transport to the laboratory.

Samples were filtered through 0.7 μm glass fiber filter (Whatman, Maidstone, England). Raw wastewater samples were only adjusted to pH 7.4 prior to icELISA assay. Surface water, treated

Table 1
IC_{50} values and cross-reactivity of the antibody toward norfloxacin and other selected fluoroquinolones.

Compound	Structure	IC_{50} (μg L^{-1})	Cross-reactivity (%)
Norfloxacin		2.2	100
Ofloxacin		>5000	<0.1
Ciprofloxacin		>5000	<0.1
Enrofloxacin		>5000	<0.1
Pefloxacin		370	0.6
Enoxacin		>5000	<0.1
Marbofloxacin		>5000	<0.1
Lomefloxacin		>5000	<0.1

wastewater, and groundwater samples were concentrated using a solid phase extraction (SPE) procedure that had been detailed elsewhere [17]. Briefly, 100 mL of the filtered sample was added with EDTA at 0.5 g L^{-1}, adjusted to pH 4.2, and loaded onto an HLB cartridge (Waters, Milliford, MA, USA) at about 5 mL min^{-1}. The cartridge had been preconditioned successively with 5 mL of methanol and 5 mL of ultra-pure water. After sample passage, the cartridge was washed with 2 mL of 10% methanol solution and vacuum dried for 10 min. The cartridge was then eluted with 5 mL of methanol. The eluent was brought to dryness under a gentle flow of high

purity nitrogen. The extracts were re-dissolved into appropriate volumes of PBS buffer, leading to enrichment factors of 5–10 for surface water and treated wastewater and 100 for groundwater.

2.8. LC–MS/MS analysis

Chemical analysis of norfloxacin was also undertaken using LC–MS/MS. An aliquot of the filtered sample (100 mL of wastewater and 400 mL of the other water samples) was spiked with 50 ng of ciprofloxacin-d_8 as the internal standard followed by enrichment with SPE as described above. The extract was finally reconstituted in 1 mL of methanol and filtered through a 0.22 μm syringe filter (Anpel, Shanghai, China). LC–MS/MS analysis was performed on an Agilent LC 1200 system coupled to an Agilent 6410 triple quadrupole MS with electrospray ionization in positive mode (Agilent, Palo Alto, CA, USA). An Agilent Zorbax Eclipse XRD C_{18} column (150 mm × 3 mm, 3.5 μm particle size) was used at a flow rate of 0.25 mL min^{-1} at 25 °C. A 4.0 mm guard column (Phenomenex, Torrance, CA, USA) containing the same sorbent was pre-connected. The mobile phase was water: methanol (40:60, v/v) with 5 mmol L^{-1} ammonium acetate and 0.2% formic acid. The retention time of norfloxacin was 3.39 min.

The optimal MS parameters were detailed elsewhere [26]. The MS temperature was 100 °C. Nitrogen was used as dry gas with a flow rate of 10 L min^{-1} and temperature at 350 °C. Nitrogen was also used as the collision gas. The capillary voltage was set at 4500 V and the nebulizer pressure was 275.8 kPa. Protonated molecular ion m/z 320.1 was set as the precursor ion for norfloxacin. The product ion m/z 302.1 corresponding to $[M+H-H_2O]^+$ exhibited the highest intensity, followed by m/z 276.0 corresponding to $[M+H-CO_2]$. Data acquisition was conducted in multiple reaction monitoring mode using precursor–product ion pairs m/z 320.1 > 302.1 and m/z 320.1 > 276.0 as the quantifier and qualifier, respectively. A dwell time of 50 ms was set for each ion transition to maximize the sensitivity. Instrument control and data acquisition were managed by MassHunter Workstation. The limits of quantification for norfloxacin were 0.100 μg L^{-1} in raw wastewater and 0.020 μg L^{-1} in river water.

2.9. Recovery tests

Recovery tests were carried out by spiking norfloxacin into PBS buffer (pH 7.4), groundwater, surface water, treated and untreated wastewater at various levels from 0.05 to 10 μg L^{-1} to determine the efficiency of the icELISA assay. Samples spiked at 0.05 and 0.1 μg L^{-1} were subjected to an SPE procedure with an enrichment factor of 5 prior to icELISA, whereas samples fortified at higher concentrations (0.2, 0.5, 1, 5, and 10 μg L^{-1}) were assayed directly. Recoveries were calculated as the following:

$$\text{Recovery}\,(\%) = \frac{C_{ss} - C_{us}}{C_s} \times 100 \quad (1)$$

where C_{ss} and C_{us} are concentrations measured in the spiked sample and unspiked samples, respectively and C_s is the spiked concentration.

3. Results and discussion

3.1. Production and characterization of the antibody

To obtain a specific anti-norfloxacin antibody, it is essential to develop an effective immunoassay. As a small molecule, norfloxacin (molecular mass = 319.3) has to be conjugated with a carrier protein to elicit the immune response of an animal to produce the anti-norfloxacin antibody. Fortunately, the active carboxylic acid group presented at position 3 in the molecule of norfloxacin (Table 1) enables direct conjugation to a carrier protein [27]. BSA and OVA are among the most commonly used protein carriers [28]. An immunogen NFX-BSA and a coating antigen NFX-OVA were synthesized using the *N*-hydroxysuccinimide active ester method [25]. UV spectrum was employed to monitor the effectiveness of conjugation reaction [28]. The absorbance for NFX-BSA shows a red-shift maximum at 286 nm compared with the 272 nm maximum for norfloxacin and 273 nm maximum for BSA (Fig. 1), indicating the successful conjugation between NFX and BSA. The coating antigen NFX-OVA gives a UV spectrum similar to that of NFX-BSA. The UV spectral analysis of the hapten–protein conjugates indicated that NFX was incorporated onto BSA and OVA at about 3.2 and 2.2 mol per mol of protein, respectively.

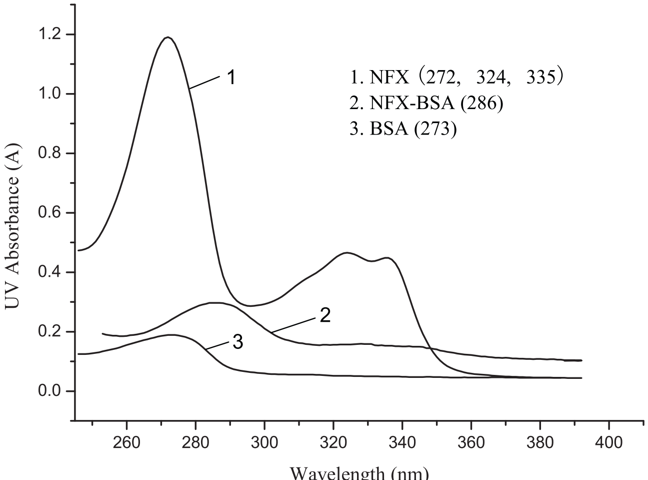

Fig. 1. UV spectra of NFX, NFX-BSA and BSA.

Titer of the antiserum, defined as the reciprocal of the dilution that resulted in an absorbance value twice that of the blank (serum from the same rabbit before immunization), increased according to immunization times. The final purified antibody has a titer of >1,024,000.

3.2. Optimization of the icELISA

To develop a specific and sensitive ELISA, assay conditions, such as concentration of the coating antigen NFX-OVA, dilutions of the antibody and GaRIgG-POD, selection of the blocking reagent, incubation time and temperature were optimized. Criteria used to assess the optimization included maximum absorbance (B_0), linear range, IC_{50} value, and limit of detection (LOD).

Accordingly, concentrations of coating antigen NFX-OVA from 0.004 to 10 μg L^{-1} in combination with dilutions of antibody NFX-BSA from 1:500 to 1:1024000 and dilutions of GaRIgG-POD from 1:500 to 1:20000 were tested using a checkerboard procedure [23]. The optimal combination of the immunoreagents was a coating antigen NFX-OVA concentration at 0.16 μg mL^{-1} with dilutions of 1:128000 for the antibody and 1:10000 for GaRIgG-POD, producing a maximum absorbance of around 1 in the absence of an analyte.

The effectiveness of the incubation for both the coating antigen and the immunoreaction was examined according to the linear range, IC_{50} value, and limit of detection (LOD). Different incubation conditions for the coating antigen (overnight at 4 °C, 60 min and 100 min at 37 °C) were tested. No significant difference was observed in incubation effectiveness when the plates were coated overnight at 4 °C or for 100 min at 37 °C. However, a high IC_{50} value (>20 μg L^{-1}) was found when the plates were coated at 37 °C for 60 min. Therefore, the plates were finally coated for 100 min at 37 °C. Immunoreaction periods of 30 min, 60 min and 120 min were

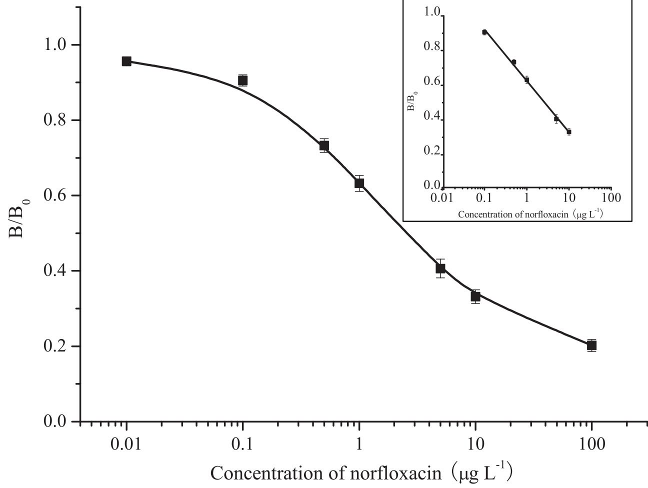

Fig. 2. Optimized calibration curve of norfloxacin generated in PBS buffer. Standard deviations ($n = 16$) are indicated as error bars.

tested at 37 °C. The result showed that 60 min of immunoreaction gave the best performance, which generated the lowest IC_{50} value and LOD and the maximum span of linear range.

The blocking buffer should be carefully selected because it is used to prevent non-specific absorbance [29]. Two blocking reagents (OVA and casein) prepared with PBS at a concentration of 1% and a Kem-En-Tec synthetic blocking buffer were tested for their blocking capacity. The Kem-En-Tec synthetic blocking buffer generated the lowest background signal (<5%) and therefore was selected as the blocking buffer in this study.

3.3. Method performance

3.3.1. Stability and sensitivity

The stability of the assay was tested by running the icELISA procedures for 16 individual times over a two-month period. Fig. 2 shows the average calibration curve generated from 16 assays. The relative standard deviation (RSD) of the measured absorbance for sixteen replicates at each standard concentration was from 1.1 to 2.5%, indicating the stability of the icELISA.

IC_{50} value is a key criterion for evaluating the sensitivity of ELISA. In this study, the calibration curve was constructed from 0.01 to 100 $\mu g\,L^{-1}$, with a linear range from 0.1 to 10 $\mu g\,L^{-1}$ ($r^2 = 0.99$, Fig. 2). The average IC_{50} value was 2.2 $\mu g\,L^{-1}$ and the LOD at a signal-to-noise ratio of 3 was 0.016 $\mu g\,L^{-1}$, suggesting that the established icELISA was highly sensitive.

3.3.2. Specificity

The CR values of ofloxacin, enrofloxacin, ciprofloxacin, enoxacin, marbofloxacin and lomefloxacin were all less than 0.1%. CR value of pefloxacin was slightly higher, which was 0.6% (Table 1). These results indicated the specificity of the generated antibody.

Previous research has revealed that the substituents located in quinolone rings may influence the cross-reactivity of antibody [27,30]. All fluoroquinolones possess a nitrogen-containing, six-membered heterocyclic aromatic ring, a carboxylic group at position 3, a ketone group at position 4 and a fluorine substituent at position 6 (Table 1). Substituents at positions 1, 7 and 8 differ by compound. Huet et al. [21] suggested that substituent at position 7 is the governing structural factor determining the binding affinity of fluoroquinolone molecules to anti-norfloxacin antibody and anti-sarafloxacin antibody. The substituent at position 8 in the structure of gatifloxacin was also believed to be a contribution to the high specificity of anti-gatifloxacin antibody [28]. The importance of substitution at position 1 in fluoroquinolone structures for antibody binding has also been stressed previously [27,30]. The poor affinity (<0.1%) of the produced anti-norfloxacin antibody toward ofloxacin, enrofloxacin and marbofloxacin seems reasonable due to different substituents at positions 1 and 7 in the structures of these compounds from those of norfloxacin. Enoxacin and lomefloxacin have an ethyl group at position 1 as norfloxacin, however, enoxacin has a nitrogen substituent and lomefloxacin has a fluorine substituent at position 8. A molecular model and an electronic property of global minimum energy conformations for enoxacin and lomefloxacin have confirmed that change at position 8 could lead to significant changes in molecule shape and electrostatic features [27]. This may explain their low CR values (<0.1%). However, it is difficult to explain the low recognition of ciprofloxacin (<0.1%) and pefloxacin (0.6%) by the antibody because both compounds are quite structurally similar to norfloxacin. The only difference between the molecular structures of ciprofloxacin and norfloxacin is the substituent at position 1, which is a cyclopropyl group for ciprofloxacin but an ethyl group for norfloxacin. Pefloxacin and norfloxacin differ in their structures by the substituent at position 7 (Table 1). One of the three broad-spectrum antibodies produced by Wang et al. [27] using the norfloxacin hapten also showed poor affinity to pefloxacin (<0.01%) but moderate affinity to ciprofloxacin (24%). Overall, the low IC_{50} value (2.2 $\mu g\,L^{-1}$) and poor cross-reactivity against other fluoroquinolones demonstrate the sensitivity and selectivity of the developed assay for the determination of norfloxacin.

Table 2
Recovery of norfloxacin determined by icELISA in spiked water samples.

Samples	Spiking concentration ($\mu g\,L^{-1}$)	Recovery (%)	Relative standard deviation (%)
PBS buffer ($n = 4$)	0.05[a]	82	4
	0.1[a]	85	4
	0.5	90	6
	1	93	4
	5	108	5
	10	101	9
Ground water ($n = 4$)	0.05[a]	84	5
	0.1[a]	84	4
Treated wastewater ($n = 4$)	0.05[a]	86	5
	0.1[a]	84	5
Wastewater ($n = 4$)	0.2	96	4
	0.5	105	4
Surface water ($n = 8$)	0.1[a]	85	3
	0.05[a]	86	3
	0.2	77	3
	0.5	74	4

[a] Sample was enriched by solid phase extraction prior to icELISA.

3.3.3. Accuracy

Recoveries of norfloxacin from spiked PBS buffer at concentrations of 0.5, 1, 5, and 10 $\mu g\,L^{-1}$ ranged from 90 to 108%, with RSDs of 4–9% ($n = 4$). Satisfactory recoveries were achieved for norfloxacin from wastewater (96–105%) and surface water (74–77%) spiked at 0.2 and 0.5 $\mu g\,L^{-1}$ with good reproducibility (RSD ≤ 4%). Good and constant recoveries (82–86%) were obtained at low spiking levels (0.05 and 0.1 $\mu g\,L^{-1}$) from various waters when the samples were treated with SPE prior to the icELISA (Table 2). The recoveries from spiked surface water samples at 0.05 and 0.1 $\mu g\,L^{-1}$ were even slightly higher than those at 0.2 and 0.5 $\mu g\,L^{-1}$, probably due to the reduction of matrix interference by the SPE procedure.

3.4. Analysis of water samples

The icELISA assay has been applied to determine norfloxacin in groundwater, surface water (river water and lake water), treated

Table 3
Concentrations of norfloxacin in water samples determined by icELISA and LC–MS/MS.

Sample	Concentration ($\mu g\,L^{-1}$)	
	ELISA ($n = 4$)	LC–MS/MS ($n = 2$)
Wastewater 1	0.13 ± 0.03	0.134 ± 0.008
Wastewater 2	0.22 ± 0.06	0.239 ± 0.040
Wastewater 3	0.18 ± 0.07	0.207 ± 0.011
Treated wastewater	0.04 ± 0.01	0.025 ± 0.006
River water	0.08 ± 0.03	0.090 ± 0.012
Lake water	<LOD[a]	<LOD[b]
Groundwater	<LOD[a]	<LOD[b]

LOD: limit of detection.
[a] LOD is 0.016 $\mu g\,L^{-1}$.
[b] LOD is 0.008 $\mu g\,L^{-1}$.

wastewater and municipal wastewater samples collected from Guangzhou, China. Norfloxacin was not detected in the groundwater and lake water samples. However, it was detected at 0.13–0.22 $\mu g\,L^{-1}$ in municipal wastewater, 0.04 $\mu g\,L^{-1}$ in a treated wastewater, and 0.08 $\mu g\,L^{-1}$ in a river water sample (Table 3). Good agreement ($r^2 = 0.983$) was observed between the results obtained by the icELISA and LC–MS/MS, further confirming the reliability of the icELISA.

4. Conclusion

A specific polyclonal antibody was obtained and a sensitive indirect competitive enzyme-linked immunosorbent assay was developed for determining trace amounts of norfloxacin in various waters. In PBS buffer, the IC_{50} value and LOD of the assay were 2.2 $\mu g\,L^{-1}$ and 0.016 $\mu g\,L^{-1}$, respectively. The icELISA was applied to determine norfloxacin in raw and treated wastewater, surface water and groundwater samples. Good agreement of the results was obtained by the icELISA and LC–MS/MS, suggesting that the developed icELISA can be a cost-effective, fast, and reliable approach for monitoring norfloxacin in aquatic environment.

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